

CONCENTRATED DAIRY CULTURES

A STUDY ON THE PREPARATION, PRESERVA-
TION AND USE OF MESOPHILIC, MIXED
SPECIES LACTIC STARTER CONCENTRATES

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CONCENTRATED DAIRY CULTURES - A STUDY ON THE PREPARATION, PRESERVATION
AND USE OF MESOPHILIC, MIXED SPECIES LACTIC STARTER CONCENTRATES

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This thesis is based on the following communications, referred to
in the text by their Roman numerals:

- I H.-E. Pettersson, Studies on Batch Production of Bacterial Concentrates from Mixed Species Lactic Starters. 1975. Applied Microbiology 29 (2), 133-140.
- II H.-E. Pettersson, Growth of a Mixed Species Lactic Starter in a Continuous "pH-stat" Fermentor. 1975. Applied Microbiology, in press, 29 (4), 000.
- III H.-E. Pettersson, Preservation of Mixed Species Lactic Starter Concentrates by Freezing and Lyophilization Methods. 1975. Milchwissenschaft, in press, 30 (10), 000.
- IV H.-E. Pettersson and G. Sjöström. Accelerated Cheese Ripening: A Method for Increasing the Number of Lactic Starter Bacteria in Cheese without Detrimental Effects to the Cheese-making Process and its Effect on the Cheese Ripening. J. Dairy Res., submitted for publication.

INTRODUCTION

Use of starter cultures in the food industry

Food fermentations, traditionally performed by spontaneous growth and action of microorganisms developing under the specific conditions existing in the food, are nowadays often carefully controlled microbial processes for which selected cultures of microorganisms are used (1, 2). These so-called starter cultures have been developed from the microorganisms that grew on or in the fermented food spontaneously by the conditions developing in the food. The transference of starter material to the food being fermented was originally spontaneous by infections from several sources. Later on a small part of the previously fermented food was used to start the subsequent food fermentation, and this method was then developed further, with the aid of the increased knowledge in microbiology and biochemistry of this century, by using selected cultures of microorganisms to give products with characteristic and preferred properties.

The microbial cultures may be added as pure cultures or mixed cultures. In table 1 starter cultures for the manufacture of some important fermented foods are listed (1 - 13). Many of these cultures are nowadays commercially available from special laboratories.

Table 1. Use of starter cultures in the food industry.

Fermented food	Type of culture used	Microorganisms
Cheese		
A. Most Swedish cheese varieties	mixed species	<i>S. cremoris</i> , <i>S. lactis</i> , <i>S. diacetylactis</i> ^a , <i>L. citrovorum</i> ^b
B. Cheddar cheese	mixed strain, single strain	<i>S. lactis</i> , <i>S. cremoris</i>
C. Mold cheese	mixed species + pure culture (mold)	<i>Penicillium</i> sp.
D. Swiss cheese	mixed species + pure culture	<i>S. thermophilus</i> , <i>Lactobacillus</i> sp. + <i>Propionibacterium shermanii</i>
Butter	mixed species	<i>S. lactis</i> , <i>S. cremoris</i> , <i>S. diacetylactis</i> ^a , <i>L. citrovorum</i> ^b
Buttermilk	mixed species	<i>S. lactis</i> , <i>S. cremoris</i> , <i>S. diacetylactis</i> ^a , <i>L. citrovorum</i> ^b
Yoghurt	mixed species	<i>S. thermophilus</i> , <i>Lb. bulgaricus</i>
Sausage	pure culture mixed species	<i>Pediococcus</i> sp., <i>Lactobacillus</i> sp., <i>Micrococcus</i> sp.
Beer	pure culture	<i>Sacch. carlsbergensis</i> <i>Sacch. cerevisiae</i>
Wine	pure culture	<i>Sacch. cerevisiae</i> var. <i>ellipsoideus</i>
Vinegar	mixed strain	<i>Acetobacter</i> sp.
Soy sauce	pure culture mixed species	<i>A. soyae</i> , <i>A. oryzae</i> , <i>Pediococcus</i> sp., <i>Lactobacillus</i> sp., <i>Sacch. rouxi</i>

^a*S. diacetylactis* = *S. diacetylactis* (8ed Bergey)^b*L. citrovorum* = *L. cremoris* (8ed Bergey)

Dairy starter cultures

A. Composition of starters

Starter cultures used for the production of fermented dairy products include cultures of bacteria, mold and even yeast (Kefir, Kumiss (1, 2)) according to table 1. The cultures of mesophilic starter bacteria, of primary interest for this study, may be classified (8) as summarized in table 2.

Table 2. Composition of mesophilic starter cultures used in the dairy industry.

- I Single strain starters
 - (a) *Streptococcus lactis* or *S. cremoris*
 - (b) *S. diacetylactis* or *Leuconostoc citrovorum*
- II Mixed strain or mixed species starters
 - (a) *S. lactis* or *S. cremoris* or both
 - (b) *S. lactis*, *S. cremoris* and *L. citrovorum*
 - (c) *S. lactis*, *S. cremoris*, *S. diacetylactis* and
L. citrovorum
 - (d) *S. lactis*, *S. cremoris* and *S. diacetylactis*
 - (e) Several strains of *S. diacetylactis* or
L. citrovorum

Starters according to group I (a), II (a) and eventually II (b) are mainly used for cheddar cheese production. The multiple mixed species starters used for cheese, butter and buttermilk in Sweden as well as in many other countries belong to group II (c) (8, 14, 15), where the content of aroma bacteria is often dominated by strains of *S. diacetylactis*.

B. Important properties of mesophilic starter cultures

Lactic starter cultures, growing in fermented dairy products, act to give the desired taste, aroma, texture and as a preservative by the production of certain metabolites (lactic acid, CO₂, diacetyl etc.) and/or by enzymatic changes of the raw material brought about by the enzyme content of the bacteria. Properties important for mesophilic mixed species starter cultures used for the production of various fermented dairy products are summarized in table 3.

Table 3. Properties important for mixed species starter cultures.

1. Lactic acid producing activity (10, 17)
2. Aroma formation (diacetyl) (8, 17, 18)
3. CO₂-production (17, 19)
4. Proteolytic activity (17, 20, 21)
5. Other enzyme activities important for cheese maturation (17, 20, 21, 22)
6. Phage resistance (8, 10, 23)
7. Stability and heat resistance (15, 16, 24, 25)

For the majority of fermented dairy products the properties listed in table 3 are of varying importance, and could even be harmful to some products if too pronounced (for example CO₂-production in certain types of cheese), but mostly these are properties of utmost importance serving as criteria for the selection of starter cultures for different products (17).

In mixed species/strain starters these properties are the resultant of a complex series of metabolic reactions by the ecological system of the food being fermented. *S. cremoris* and *S. lactis*, which are the dominant species in these starters, are responsible for lactic acid production (8), and in ripened products (cheese) they also contribute to the amount of ripening enzymes (20, 26, 27) (proteases and others). The aroma forming bacteria, *S. diacetylactis* and *L. citrovorum*, produce apart from minor amounts of lactic acid and other volatile acids, diacetyl and CO₂ (8). They should also contribute to some extent to the maturation of cheese. All changes in the composition of such a complex starter due to various growth conditions, infections by other microorganisms and bacteriophages will bring about a change in the net reactions in the food, leading to fermented products with abnormal properties. Therefore it is of utmost importance that the dairy industry, especially today with its increasing plant sizes, can obtain starter cultures with constant and stable properties from commercial starter laboratories.

C. Production of starters

Production of starter cultures involves both the selection, preparation and eventually the preservation of these cultures at the starter labo-

ratory, their distribution, and the handling of these cultures for the production of fermented products at the dairies.

Mixed species starter cultures are often old cultures grown in mixtures, or mixed from several pure cultures at the starter laboratories (16), selected according to their specific properties. Starter cultures are distributed from commercial laboratories as (a) liquid cultures, (b) lyophilized (freeze-dried) cultures, (c) frozen cultures or (d) frozen concentrated cultures. The use of the different types of cultures in the Scandinavian countries in 1971 was as follows (28):

Liquid cultures	97%
Frozen culture concentrates	2%
Lyophilized cultures	1%

Since then there has been an increased tendency toward the use of frozen culture concentrates in the dairy industry in Sweden (28), as well as in other countries (29, 30). The advantages and disadvantages of the various types of starter cultures are summarized in table 4 (29, 31-33).

Table 4. Advantages and disadvantages of various starter techniques.

	Advantages	Disadvantages
Liquid cultures	1. Familiar technique	1. Loss in activity
	2. Several subcultures in the product milk	2. Several subcultures
	3. Possibility of choosing cultures	3. Risks for infections
		4. Daily handling
Lyophilized liquid cultures	1. Easy to handle	1. Low activity
	2. Easy transportation	2. Several subcultures
	3. Good storage stability	3. Risks for infections
	4. Possibility of choosing cultures	4. Daily handling
Frozen concentrated cultures	1. High and stable activity	1. Transportation and handling
	2. One or no subculture	2. No possibility of choosing cultures
	3. Minimum risks for infections	
	4. Labour saving	

As seen from table 4, most of the disadvantages associated with the use of liquid cultures (even frozen or lyophilized unconcentrated cultures) could be overcome by the new technique of frozen (eventually lyophilized) concentrated starters. This technique involves no bacteriological changes of the starter material, but merely a new way of producing and preserving starters and thereby simplifying their use at the dairies.

A great deal of research work has been done during the last 10 years on methods for the preparation and preservation of such starters (34, 35). They all involve one or more of the following principal methods:

- (a) Mechanical concentration (centrifugation)
- (b) Continuous or half continuous neutralization of lactic acid
- (c) Neutralization and centrifugation

By these methods the concentration of bacteria can be increased 10 - 100 times compared to a normal outgrown milk culture. Starter concentrates prepared according to the above methods continuously or batchwise (34, 35) should then be frozen at a sufficiently low temperature (or lyophilized) to preserve the activity until the time for use. Most of the detailed studies on starter concentrate preparation have been done by batchwise cultivation of pure cultures, primarily used for cheddar cheese production (36-39).

Preservation by freezing in liquid nitrogen (-196°C) has been shown to be the best method for preserving starter concentrates (38, 40-43), but higher temperatures (-20°C - -40°C) have been shown to be sufficient for shorter storage time (44-46).

Frozen starter concentrates are nowadays used in the dairy industry mostly to inoculate the bulk starter, but some promising investigations have been performed on the use of these highly concentrated starters to inoculate the process milk directly (37, 46-49). The advantages that may be achieved by using starter concentrates as a direct inoculum are summarized in table 5.

Table 5. Advantages of the "direct inoculation" method.

1. Smaller risks for infection
(microorganisms, phages)
2. Identical inoculum from day to day
3. Even product quality
4. Labour saving
5. No special rooms required for bulk starter preparation.

Aim of the project

The formulation of the aim of this project is based on the facts, summarized above, that detailed studies of production and preservation of mixed species starter concentrates were lacking in the literature and that direct inoculation with concentrated starters had many promising advantages over the use of bulk starter. Furthermore it should be possible to use starter concentrates with high cell concentrations in an unconventional way, as an enzyme source in the preparation of ripened products (cheese).

The aim of this project was (a) to study the preparation of concentrated mixed species starters, directly from commercial mixed species starters, by batchwise and continuous cultivation methods, to optimize the preparation and (b) to study and evaluate the effect of different preservation methods on these concentrates with the intention of using them as a direct inoculum in cheese making. (c) A third part of this project was undertaken to study a method of using starter concentrates as an extra enzyme source to accelerate cheese ripening.

MATERIALS AND METHODS

Cultures

The following commercial, mixed species starter cultures were used in this study: Flora Danica (FD) and Flora Danica special (FDs) obtained from Flora Danica Laboratory, Odense, Denmark; Christian Hansen normal (CH) and Christian Hansen special (CHs) were obtained from Christian Hansen's Laboratory, Copenhagen, Denmark, and T 27 was obtained from Viesby Maelkerei-Laboratory, Tonder, Denmark. They all contained strains of *S. cremoris/lactis*, *S. diacetylactis* and *L. citrovorum* (BD-type starter). Stock-cultures of these strains were kept in liquid nitrogen to keep the same bacterial material throughout an investigation (I).

Lactobacillus bulgaricus CNRZ 36, *L. helveticus* CNRZ 303, 32 and *Streptococcus thermophilus* CNRZ 21 were obtained from Centre National de Recherches Zootechniques, Jouy-en-Josas, France.

Media

Three different media were used in the cultivation experiments in this study:

- (a) Skim milk medium, with nine percent reconstituted non-fat milk solids (NFMS) (I-IV),
- (b) Whey medium was prepared according to a procedure by Pettersson (I-III),
- (c) Tryptone-lactose-yeast extract medium was prepared according to Bergère (50) (I-III).

Cultivation techniques

In most experiments starters were cultivated in a 1 l laboratory fermentor purchased from AB Biotec, Hägersten, Sweden. In some experiments, a 10 l laboratory fermentor, purchased from Marubishi Ltd, Tokyo, Japan, was used. Both fermentors were equipped with controls for pH (Radiometer A/S, Copenhagen, Denmark), temperature and stirring speed.

In the batch cultivation experiments, sterile medium in the fermentor was inoculated (1-2% (v/v)) with an active starter culture. Temperature and stirrer speed were kept constant throughout the cultivation. Constant pH was maintained by neutralizing the medium continuously with either 5 M NaOH or 20% (v/v) NH_4OH to a pre-set value.

For the pH-stat continuous cultivation experiments, the 1 l glass fermentor was connected to a medium reservoir via a pH-controlled peristaltic pump (LKB-Beckman Instrument AB, Vällingby, Sweden) and to a spent medium reservoir via an overflow tube (II, Fig. 1). The continuous culture was inoculated as a batch-culture, but pre-set pH-values were kept constant by adding fresh medium by the peristaltic pump. By setting the speed of the pump at a sufficiently low value, the flow was kept almost continuous.

Samples for analyses during the cultivation experiments were taken through a sampling port on the top of the fermentor (batch) or through a sampling line (continuous).

Mechanical concentration

Starters cultivated batchwise or continuously were further concentrated by a centrifugation procedure. Previously cooled ($+5^{\circ}\text{C}$) starter suspensions was distributed in glass tubes or stainless steel tubes and centrifuged in a Sorvall SS-3 superspeed centrifuge (Ivan Sorvall Inc., Newtown, Connecticut, USA) at 34 000 g for 10 min. In some experiments, in which the working volume of the suspension was too large, it was centrifuged in the same centrifuge equipped with a continuous centrifugation rotor with the same centrifugal force.

Preservation methods

Starter concentrates were frozen and stored at the following temperatures: Liquid nitrogen at -196°C , methanol at -30°C (Ultra-Kryomat, Messgeräte-Werk-Lauda, Western Germany), and at -25°C in air with an ordinary freezing cabinet (Electrolux, Stockholm, Sweden). Before freezing, starter concentrates were, in some experiments, mixed with cryoprotective agents (1:1) such as reconstituted skim milk (NFMS), glycerol (50% (v/v) water solution), skim milk + glycerol (4:1) (51) or 15% (v/v) lactose solution (44), and in some experiments neutralized with 5 M NaOH (III).

Freeze-drying of concentrates was performed in 7% lactose (44), 0.06 M mono-sodium glutamate or 0.06 M arginine-mono-hydrochloride (52) with a freeze-drying apparatus (Hetofrig CB6 and Heto CD11, Heto Birkerød, Denmark). Freeze-dried material was stored in evacuated ampoules or in glass vials in N_2 or air at $+20^{\circ}\text{C}$ or $+5^{\circ}\text{C}$ (III).

Partial inactivation of starter concentrates

Starters or starter bacteria (mixed species starter or thermophilic bacteria) cultivated at constant pH were partly inactivated by heating at temperatures from $50 - 80^{\circ}\text{C}$ with a holding time of 15 sec in a glass apparatus constructed from standard Pyrex glass apparatus. Starter suspension ($10^9 - 10^{10}$ cells/ml) was pumped by a peristaltic pump (LKB-Beckman Instrument AB, Vällingby, Sweden) through the heating apparatus at a constant flow rate (80 - 90 ml/min) (IV).

Cheese-making

Two Swedish cheese varieties were made in small scale cheese-vats (7 - 50 l), one open texture variety ("Hushålls"-cheese), and one

closed texture variety with eye-formation ("Herrgård"-cheese) (III, IV). In one series of experiments, direct inoculation of frozen starter concentrate was tested and compared to normal bulk starter inoculation of the cheese-milk (III).

Starter bacterial suspensions partly inactivated by heat were used as an extra inoculum, along with a normal mesophilic mixed species starter, in experiments to study the effect of an increased number of bacteria in cheese on cheese-ripening velocity (IV). These experiments were also performed in parallel, with or without the extra inoculum of inactivated bacteria, on the same lot of milk.

Analytical methods

Total amount of bacteria was determined by a pour-plate technique on calcium-citrate agar (53) and by direct microscopic counts according to Skar (54). Counts in cheese were performed on a homogenized cheese slurry (1% sodium-citrate at 45°C) (IV).

Determinations of total amount of bacteria in cheeses were also done by total DNA-analyses of the homogenized cheese according to Burton (55) and Ohimiya and Sato (56) (IV).

The bacterial composition of mixed species starters or starter concentrates was determined by a method of Nickels and Leesment (53).

Lactic acid producing activity of starters, starter bacteria and concentrates was measured by pH recordings of skim milk samples (9.1% NFMS) inoculated to a pre-determined concentration with the bacteria and incubated with continuous stirring at constant temperature (I - IV). Relative activities were determined according to a procedure by Bergère (50).

Proteolysis of starter bacteria was determined by a method of Hull (57) modified by Citti *et al.* (58) (I - IV). Starter bacterial proteolysis was also determined by measuring the increase in TCA soluble nitrogen by the Kjeldahl method (IV).

Production of diacetyl by mesophilic, mixed species starters or concentrates was determined by a method of Owades and Jakovac (59), modified for milk cultures by Pack *et al.* (60) (I - III).

Gas production (CO_2) by strains of *L. citrovorum* and *S. diacetylactis* in the mixed species starters or starter concentrates was determined by the Warburg technique (61), modified for concentrated lactic starters by the author (I - III).

pH, citric acid (62) and total amount of bacteria (53) were determined during the first 24 hr of the cheese-making experiments. 24-hr cheeses were analysed for water, pH, fat, lactose and protein according to standard routine methods. Cheeses stored for various periods of time were analysed for volatile acids according to Harper *et al.* (63), and for protein breakdown as TCA soluble N, PTA soluble N (64) relative to total nitrogen by the Kjeldahl method (III, IV). In some experiments, amino nitrogen was determined by formol titration (65).

Cheeses were also judged according to flavour intensity and texture by a panel of cheese experts, who graded the cheeses in points on a hedonic scale of 1 - 5.

RESULTS AND DISCUSSION

Batchwise or continuous growth of mixed species starter cultures at constant pH (I, II)

One pre-requisite condition for all growth studies on the complex mixed species cultures of commercial origin used in this study was that the culture material could be kept constant throughout a total series of experiments. This was achieved by freezing the inoculated starter culture in liquid nitrogen (-196°C). By this method, stock cultures with unchanged properties compared to the unfrozen starter could be kept for at least 2 years (I, table 1). This beneficial effect of freezing in liquid nitrogen was also verified in a later part of this work (III).

The first part of this study was undertaken to evaluate growth behaviour of mixed species starters in two different fermentation systems working at constant pH, with the intention of making concentrated starters by an additional concentration procedure (centrifugation).

Investigations of optimal growth conditions for mesophilic single strain lactic cultures in batch fermentations at constant pH (36, 38, 39, 66, 67), as well as in pH-stat continuous fermentations (68-71), have

previously been carried out by others. Results and experiences from these studies served as a background for the planning of the experiments in the present work.

Specific growth rate and maximum cell yield increased with temperature from 20°C to 30°C when mixed species starter FDs 0172 was grown batch-wise at constant pH in either of the three media examined (I, Figs. 1 - 3). The constant pH value (5.5 - 7.0) in batch cultivations was not found to significantly influence the growth of the total cell mass of the starter. Compared to a static milk culture, the total amount of bacteria in the outgrown pH-stat culture increased by a factor of 4 - 20 for all starters examined (I, table 2), in good agreement with the results of others (11, 38, 44, 66). Increased growth rate and maximum cell yield compared to skim milk medium was found in whey medium, and especially in tryptone medium (I, Figs. 1 - 3, Table 2), by batch cultivation. Apart from effects of constant pH and temperature on starter growth, the influence of different neutralizers was examined (I, Table 3). NH_4OH was not found to increase growth rate and maximum count in this study compared to NaOH , which is contradictory to the results of previous workers (35, 37, 38, 72). But since these studies were carried out on single strain cultures comparisons may not be valid.

To get some further information on the growth behaviour of the mixed species starter in batch fermentations, the behaviour of the main strain components (*S. lactis/cremoris*, *S. diacetylactis* and *L. citrovorum*) was examined according to Nickels and Leesment (53). By these refined examinations all parameters, *i.e.* temperature, pH, medium and neutralizer, were found to influence strain balance. Growth medium, different from milk, was found to have a detrimental effect on the changes in bacterial balance during growth at constant pH (I, Figs. 5 and 6, Table 2).

By the strain balance analyses, the neutralizer was also found to have a marked effect (I, Table 3). NH_4OH was found to preserve strain balance much better than NaOH , which caused a change in the bacterial balance in favour of the aroma bacteria. This could to some extent be explained by the mechanism proposed by Zarlengo and Abrams (74) and Peebles *et al.* (38) that NH_4OH (free NH_3) could more easily penetrate the cell wall and thereby control the internal pH more accurately. This effect should be of special importance for the dominant lactic acid producing

strains (*S. cremoris*) with low internal pH:s at times, depending on the vigorous lactic acid production during growth. Maximum growth was achieved in the interval between 10 - 16 hrs in this study, depending on pH and temperature. At this time, various amounts of lactose still remained unfermented in the media (20 - 40%). Lactate (39, 66, 67) and D-leucine (75) have been shown to have inhibitory effects on lactic starter growth. The inhibitory effect of lactate was found to be very complex in nature, varying with all parameters examined (I, Fig. 7). No explanation for this could be given, but it could indicate that other (auto-)inhibitors produced by the bacteria under certain conditions (*e.g.* D-leucine) are involved.

The continuous cultivation method used in this study, with the principle function of neutralizing lactic acid produced in the growth vessel by the addition of fresh medium, is often referred to as a pH-stat continuous fermentation (68, 76), or more generally a bacteriostat continuous fermentation. Under continuous operation, the specific growth rate (μ) is numerically equal to the dilution rate (D) (77, 78), where D is defined as:

$$D = f/v$$

f = flow rate, ml/hr

v = cultivation volume, l

Mixed species starter, FDs 0172, used as a test culture in the experiments concerning continuous pH-stat cultivation, was cultivated continuously for up to 120 hrs at various constant pH values at 25°C, and in the same three media used for the batch fermentations. Dilution rates and specific growth rates under steady-state operation increased with increasing pH (pH 5.5 - 6.3) in all media (II, Tables 1 - 3, Figs. 1 - 2). In tryptone medium, growth rates were higher than in the other two media, but the total number of cells was lowered, due to the better buffering capacity of milk and whey medium and the higher growth rates in tryptone medium shown by the batch studies.

Specific growth rates for the continuous cultivations were overall lower than the corresponding values for batch cultivations at constant pH. At higher pH values (≥ 6.0) these differences diminished markedly. The cause of this could be the more accurate control of pH by a neutralizer (NH_4OH , NaOH) compared to the dilution effect on pH when

cultivating continuously by the method described, thus causing a more harmful effect on the bacteria at lower pH. Other effects such as accumulation of inhibitors (69, 70) and selection of strains could also have been involved.

Yield of bacteria, *i.e.* productivities, calculated as number of cells produced in the continuous fermentor per liter of culture volume per hour, showed maxima at pH values and specific growth rates depending on the medium of cultivation (II, Tables 1 - 3, Figs. 1 - 2). This maximum, of special importance when comparing the two cultivation methods, was greatest in skim milk medium, reflecting the better buffering capacity of this medium. The bacterial balance, as determined on calcium citrate agar (53), was quite stable and unchanged when pH-stat continuous growth was performed in skim milk or whey medium at almost any pH tested. However, at the end of the cultivation (120 hrs), a tendency toward a decrease in the amount of *L. citrovorum* could be observed in these media. When grown in tryptone medium, at pH values lower than 6.0, the bacterial balance was changed toward an increase in the proportion of strains of *S. diacetilactis*, with a corresponding decrease of strains of *S. cremoris/lactis* and *L. citrovorum*.

Effect of various growth conditions on certain metabolic activities (I, II)

The methods used for bacterial balance determinations (53) were relatively approximate, leaving changes between multiple strains of one and the same species out of control. Changes between strains of homofermentatives, *S. lactis*, *S. cremoris*, which was not detected by the method used (53), could be of great importance, leading to variations in growth and lactic acid production of a starter concentrate. Alternatives would be to complement with other selective media, for example that developed by Reddy *et al.* (79) (IV), for the selection between *S. cremoris* and *S. lactis*. This would not give much further information about this starter dominated by strains of *S. cremoris*. Another possibility would be to use a phage-typing technique according to Gilliland (73). Further information about the exact composition of the commercial starter and the corresponding phage-types is, however, needed before this sophisticated technique can be applied to the complex starters used in this study. Because of this the analyses for growth and bacterial balance had to be complemented with analyses of metabolic activities of importance for various fermented dairy products.

Lactic acid production of starter biomass, in this study measured as pH-drop versus time under standardized conditions, was found to be very sensitive to prolonged growth in batch fermentations (I, Figs. 1, 2 and 4) both in skim milk and whey medium, with a rapid decrease after the end of the logarithmic growth period. A comparison between this activity and the bacterial balance during prolonged growth showed that the decrease in activity was followed by a corresponding loss of strains of *S. cremoris/lactis* relative to the amount of aroma bacteria. Extreme sensitivity of strains of *S. cremoris* to inhibitors (lactate) was reported by Bergère and Hermier (66). This makes it very important to choose the optimal harvest time when making concentrated starter cultures by batch cultivation methods.

Growth at constant pH in tryptone medium caused an unexpectedly high lactic acid production of harvested concentrate compared to the changes in bacterial balance, probably due to changes in the balance between homofermentative strains, *i.e.* selection of strains with high activity (I, Fig. 3). Information in the same direction is also given by the high lactate concentrations necessary for inhibition of growth in this medium (I, Fig. 7).

Neutralization with NaOH instead of NH_4OH was detrimental to the lactic acid producing activity of starter biomass at the time of harvesting. This could perhaps also be explained by the different effect of neutralization by the two neutralizers as stated above (38, 74) on the bacterial cell. Lactic acid production of cells harvested from pH-stat continuous cultivation was influenced both by the medium and the constant pH, with an increase in activity with pH (II, Tables 1 - 3) in good agreement with the results of Berridge (80). At least three possible explanations could be given for this effect:

- (a) growth at lower pH (5.5 - 6.0) affected the production of proteases necessary for a normal growth rate and lactic acid production (80);
- (b) the increased inhibitory effect of lactic acid at lower pH values (I, Fig. 7) affects cells of *S. cremoris* at a higher rate; or
- (c) the increased residence time in the fermentor at lower pH caused a selection of slow lactic acid producing variants. The substrate effect could to some extent be explained by the difference in buffering capacity of the three media.

Other important cell activities of the starter concentrate, which were measured as a function of growth conditions, were diacetyl production, CO₂ production and overall proteolysis. Production of diacetyl by the starter biomass seemed to be influenced by the various citrate levels of the three media used (I, Table 2, and II, Tables 1 - 3). Such an effect was also observed by Gilliland *et al.* (41) for *Leuconostoc* species grown in broth without citrate. The lower diacetyl value for concentrates cultivated in tryptone medium, measured after 16 hrs at 22.5°C in this study, could perhaps also be attributed to the effect of this medium on bacterial balance, leading to dramatic changes in the composition of the starter with an increased proportion of *S. diacetilactis*. Such changes in the starter composition would probably cause changes in the aroma formation versus time compared to the original starter.

CO₂ production of cell biomass cultivated batchwise or continuously were parallel to changes in bacterial balance, with increased production from cells grown in tryptone medium (I, Table 2, II, Tables 1 - 3).

Proteolytic action of the cell mass was lowered by batch cultivation in tryptone medium (I, Table 2) in accordance with the results of Williamson and Speck (81), who found that the proteolytic activity of starter bacteria was lowered by growth in media richer in peptides and amino acids than milk.

Optimal conditions for the production of mixed species starter biomass (I, II)

An ideal method of cultivation for the production of starter bacteria to be further concentrated by centrifugation, and to be used primarily as a direct inoculum for the production of fermented milk products, should have the characteristics summarized in table 6.

Table 6. Some important features of an ideal cultivation method for the production of concentrated starters.

1. Good substrate utilization
2. High growth rate and max. count
3. Maintenance of unchanged bacterial balance
4. Production of starter biomass with cell activities comparable to inoculum
5. Ease of control
6. No problems with infections

In table 7 a batch cultivation of FDs 0172 at pH 6.5 (20% NH_4OH) and 25°C in skim milk medium is compared to a continuous pH-stat cultivation of the same starter at pH 5.9 and 25°C in skim milk medium. These conditions for cultivation were found to best meet the criteria stated in table 6.

Table 7. Comparison between optimal conditions found for production of starter biomass by batchwise or continuous growth methods. Mixed species starter FDs 0172.

Parameter	Method of cultivation	
	Batch	Continuous
Productivity, cells/l, hr	$4.3 \times 10^{10^a}$	5.0×10^{10}
Max. count, CFU/ml	60×10^8	9.3×10^8
Yield, CFU/l medium	6.0×10^{12}	0.9×10^{12}
Bacterial balance, % aroma bacteria	20	20
Rel. lactic acid producing activity, %	100	100

^aOverall productivity (overall growth cycle = 14 hrs).

From table 7 it can be concluded that continuous cultivation had no advantage over the batch method. Substrate utilization was low on continuous cultivation, with a low cell concentration in the medium leaving the fermentor. However, the characteristics of the starter produced were not changed by either of the two methods. Some improvements of the continuous cultivation, for example by an improved construction which allows milk medium to be used for longer runs (> 5 days), by improving the whey medium, or with a better substrate utilization by another type of continuous process (42, 82), would still make this way of producing concentrated starters an interesting alternative to batch methods.

Preservation of mixed species starter concentrates by freezing or lyophilization (III)

Between the preparation of starter biomass by methods outlined above and the preservation procedure, a further concentration was effected

by centrifugal concentration. This process gave a cell mass with a concentration of $6 - 20 \times 10^{10}$ cells/g, and was never found to influence the characteristics of the starter analysed at the time of harvesting. One pre-requisite necessary to take full advantage of the idea of concentrated starters, either for bulk starter preparation or for use as a direct inoculum, is that the concentrate can be preserved with minimum losses from the time of preparation to the time of use in the dairy.

Freezing and storage at ultra-low temperatures (-196°C) at pH $\hat{=}$ 6.0 was found to be the best method for preserving starter concentrates of such complex compositions as those used in this study. By this method, viability, strain balance and important metabolic activities such as production of lactic acid, diacetyl and CO_2 and proteolytic activity, were preserved without losses compared to the unfrozen concentrate during the entire period of examination, which varied from 3 - 13 months of storage (III, Figs. 1 - 5, Tables 1 and 2). Freezing and storage at higher temperatures (-30°C (methyl alcohol)) and -25°C (air)) caused losses in viability and activity during storage. Losses were, however, quite small on freezing in methyl alcohol (-30°C) for a storage period of up to 150 - 200 days, except for small losses in lactic acid production during the first few days and the loss in CO_2 production, which was marked even after 3 days. Losses were more evident on freezing at -25°C (air), with great losses in lactic acid and CO_2 production during the first days of storage.

Cryoprotective agents (glycerol, skim milk, lactose) improved the results to some extent at higher freezing temperatures, especially in the case of cells frozen at -25°C (III, Tables 3 and 5). Some negative effects of glycerol on cell stability during frozen storage was observed, which has also been found by Gibson *et al.* (23).

The pH of the freezing menstrum influenced the freezing stability of the concentrate very much, with a loss in lactic acid production at all freezing and storage temperatures at pH:s lower than 6.0 (III, Table 4). This pH effect, observed even at -196°C , is in contrast to the results of Baumann and Reinhold (24), who found no influence of pH on freezing stability at this temperature.

A more attractive way of preserving starter concentrates, with respect to handling and transportation, is that achieved by lyophilization. Recently, very promising results have appeared in the literature (44, 85, 86), which could make this method of preservation a real alternative to freezing.

In this work, freeze-drying of concentrated mixed species starters was studied with respect to viability, bacterial balance and important cell activities. Freeze-drying of the concentrate with 7% lactose as a protective agent, and subsequent storage of the freeze-dried powder in evacuated ampoules at $+5^{\circ}\text{C}$, preserved the concentrate for up to 9 months with about 50% loss in viability, lactic acid production and diacetyl formation. An interesting observation found in these trials was that the bacterial balance did not change markedly during storage (19 months) (III, Table 6), which makes this preservation method comparable to freezing at -196°C . The latter observation is, however, in contrast to the results of Reddy *et al.* (86), who froze or freeze-dried simpler mixtures of starter bacteria and found that freezing at -196°C preserved strain balance better than freeze-drying.

The preferred method of preservation is very much dependent on how the starter concentrate is to be used in the dairy. If the concentrate is to be used for direct inoculation of the process milk, *i.e.* as a bulk starter, very small losses in viability and cell activities can be tolerated, because such losses much be compensated by a larger volume of concentrate, which would considerably increase the costs for freezing, transportation and handling. From this point of view, only freezing at -196°C fulfils this requirement, which was also verified in this study by making a hard cheese variety ("Herrgård"-cheese) by direct inoculation of the cheese vat with a mixed species starter concentrate frozen and stored at -196°C for up to 133 days. Cheeses were comparable to controls, even with respect to taste and texture judged after 1.5 or 2.5 months of storage (III, Table 7). However, some differences in the proteolysis between experimental and control cheeses (III, Table 7) revealed an effect on proteolytic enzymes, due to this freezing method, which was not detected by analyses in milk (III, Table 2) (87, 88).

This study has shown that it should be possible to prepare and store, without significant losses, highly concentrated ($\leq 2 \times 10^{11}$ cells/g) and active mixed species starters comparable to an ordinary bulk starter preparation for cheesemaking. More research is, however, needed both on the preparation of starter concentrate and on the preservation methods coupled to large scale productions of fermented products before any definite conclusions can be drawn about the advantages of direct inoculation compared to bulk starter handling, especially with respect to the economics.

Accelerating cheese ripening by the use of concentrated cheese starter (IV)

An interesting aspect of the use of starter concentrates is based on the assumption that an increase in starter bacterial enzymes in cheese would also increase the speed of formation of important aroma compounds, leading to shorter ripening time. Attempts to shorten ripening times by increasing the number of starter bacteria in the final cheese have been made in various ways (27, 89, 90, 91) with varying results, mostly depending on the relatively small increase in starter bacteria achieved. With the highly concentrated starters of today, new possibilities to accelerate cheese ripening open up.

In this study, an attempt to accelerate cheese ripening by a method described by Erikson and Sjöström (91), here modified for concentrated starter cultures, was undertaken. A heat-treated starter bacterial suspension (batch cultivation; constant pH), with or without further concentration, was added to the cheese vat, together with an ordinary untreated starter (mesophilic, mixed species starter) at the same time (20 min before renneting), with the intention that the extra addition of starter bacteria would not interfere with the cheese making process, but would merely be incorporated into the cheese-matrix and serve as an extra source of ripening enzymes. The heat treatment was undertaken to reduce lactic acid production of the bacterial suspension to such an extent that most of the lactose would be fermented in the cheese (92) before these extra bacteria regained full activity. On the other hand, this heat treatment should not destroy important enzyme systems, especially the proteases which were studied in this work. Heat treatments of mesophilic mixed species starter suspensions

revealed that a time/temperature treatment of 58-59°C for 15 s was necessary to reduce lactic acid production sufficiently (IV, Table 1), which caused a loss in the proteolytic activity (TCA sol. N) by 20 - 30% (IV, Fig. 1) compared to an untreated starter suspension.

Thermophilic starter bacteria, used in this study as an attempt to further improve this method, showed a higher heat stability. Heating at 69°C for 15 s was necessary to achieve an acceptable reduction in acid production for a *Lactobacillus* strain (IV, Table 2). The corresponding loss in proteolytic activity (TCA sol. N) was 10 - 15% (IV, Fig. 2).

Swedish, semi-hard cheese ("Household"-cheese) was made by the method outlined. Heat-treated suspensions were added to the cheese-milk in varying amounts, and the effect on the cheese-making process, the final cheese and the cheese-ripening was studied.

An interesting observation was that the percentage incorporation of bacterial cells in cheese gradually decreased as increasing amounts of cell suspension were added. This was more evident for the cocci than for the lactobacilli (IV, Fig. 3), which was probably due to steric factors depending on differences in shape and size of these bacteria. The cell concentration in the final cheeses was increased by at most a factor of 4 - 5 compared to a normal cheese (10^9 cells/g). These amounts were found to influence neither the cheese-making process (IV, Tables 4, 5) nor the chemical composition of 24-hr cheese. The formation of various fractions of nitrogen compounds was, however, markedly increased by the increasing amount of extra bacteria in the cheese-matrix (IV, Figs. 4 - 6). This importance of starter bacteria in protein breakdown is in accordance with the results of others (26, 27). Measurements of free fatty acids, indicating lipolytic activity, revealed only small increases compared to the control cheeses.

To evaluate the effect on cheese ripening by the method outlined, organoleptic judgements were undertaken after 1 and 2 months of storage (IV, Tables 6 and 7). No differences in texture between cheeses made with extra bacteria and control cheeses were found. Judgement of flavour intensity, on the other hand, revealed that an

increased grade score was achieved in the experimental cheeses compared to controls. These results indicated that it should be possible to shorten ripening time for this type of cheese by approximately 1 month (mesophilic starter) with the same degree of ripening as an ordinary ripened cheese. A positive correlation ($r = 0.8$) was also found between PTA-soluble nitrogen, referred to as amino acids and flavour intensity, which supports Marth's (93) suggestion that amino acids act as intermediate products for certain aroma compounds.

The reason for using thermophilic starter bacteria in this study was to investigate a further improvement of this method by using bacteria with higher and different enzyme activities compared to the mesophilic starter. By using a heat-treated *Lactobacillus* strain there was a marked increase in proteolysis (IV, Figs. 5, 6) and flavour intensity (IV, Table 7) compared to cheeses made with additional mesophilic cocci. Cheeses made with extra lactobacilli often had a somewhat strange cheese flavour for this kind of cheese. Accordingly, the method might be further used for developing new cheese varieties by introducing ripening bacteria of certain selected strains and species.

Economic aspects of accelerated cheese ripening (IV)

For hard and semihard cheese varieties with long ripening times, storage costs are a major part of the cost of the mature cheese (~ 30%). To get an estimation of the economic profit from the method for accelerating cheese ripening investigated in this study, an estimation of costs for a plausible process installed at a cheese plant of average size (1 500 t cheese/year) was made. This process, installed at the dairy plant, consisted of a simple fermentor equipped with controls for pH and temperature and a plate heat exchanger (locally available) mounted with necessary controls, linings and pumps to fit in with the existing dairy equipment. The calculation was made with the assumption that a 3% (v/v) mesophilic starter bacterial suspension (1 500 l/day) would be added to the cheese milk, which is a realistic value according to the results above (IV, Fig. 4, Table 6). It was also assumed that no loss in cheese yield would result from the addition of extra starter bacteria, *i.e.* there was no cost for milk substrate used for the preparation of starter suspensions (not centrifuged). Furthermore, the wage and price levels for these cal-

culations were those of 1974. The result of this calculation of costs is shown in Table 8, and the profits from the procedure for accelerating cheese ripening, resulting from various reductions in storage time, can be read from figure 1 (94).

Table 8. Approximate annual costs for accelerated cheese ripening at an average dairy plant producing 1 500 tons of hard cheese per year.

Cost distribution	Annual cost, thousands of Sw. kronor
Buildings	4.0
Machinery	30.0
Labour costs	23.5
Energy	6.0 - ...
Costs for requisites	20.5
Costs for control	4.0
Total	88.0

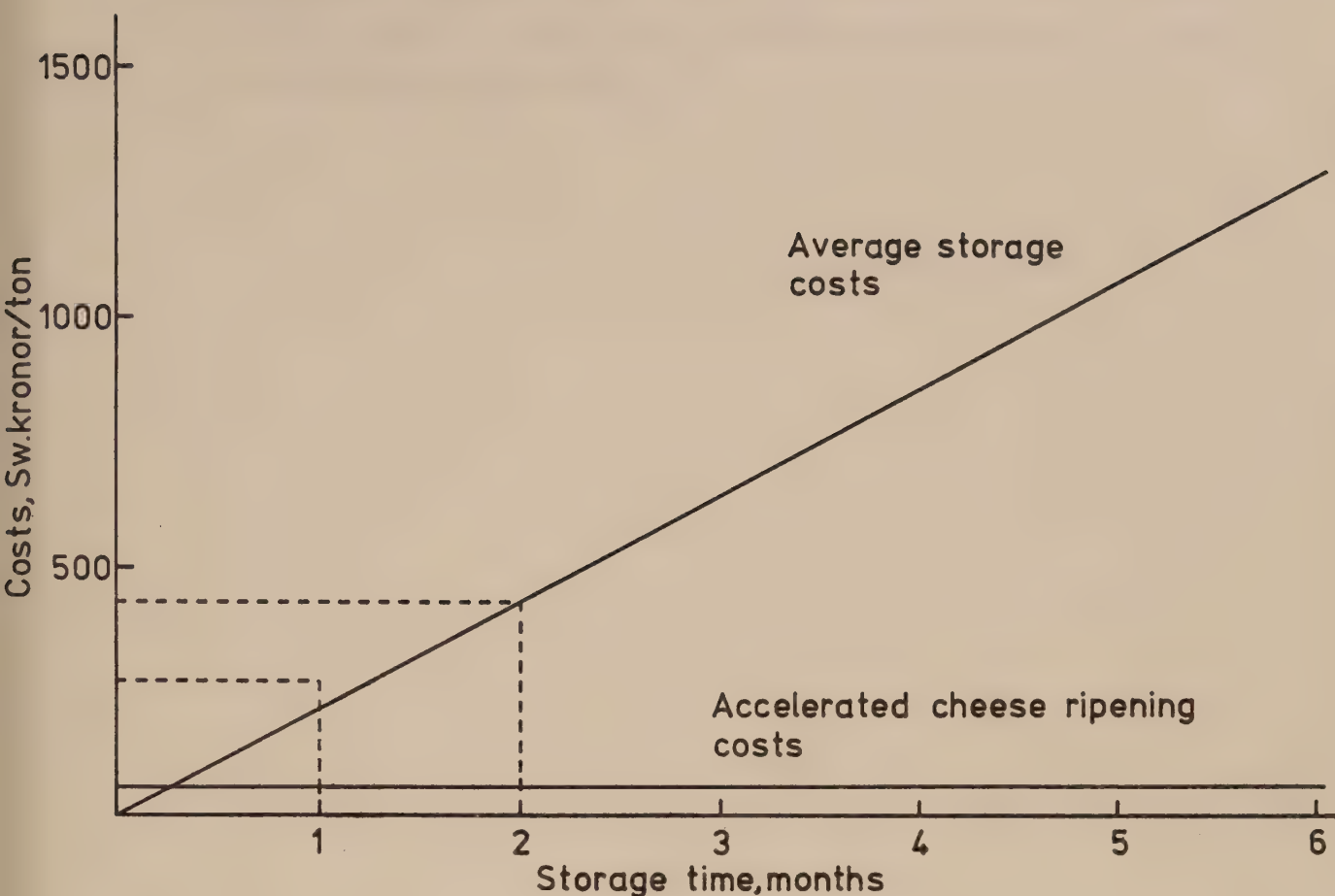


Fig 1. Costs for cheese storage and accelerated cheese ripening at an average Swedish dairy plant producing 1 500 tons of hard cheese per year.

From Figure 1 it can be seen that 1 month's storage cost for hard cheese (94) is about 3 times the costs for "accelerated cheese ripening" according to the method outlined, *i.e.* by lowering storage time by one month, 2/3 of the storage costs for one month could be saved. By selecting starter bacteria with better ripening capacity (proteolysis etc.), greater reductions in storage time could be achieved, which in this study was demonstrated by the use of lactobacilli (IV, Table 7). Such an improvement would make this method even more profitable.

The conclusion to be drawn from this is that the economic feasibility of this method for shortening ripening times is evident as based on the results of the small-scale experiments. Modifications of the

process not foreseen in this study, can, however, be shown necessary when the process is scaled up to a normal industrial scale, leading to somewhat increased costs.

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